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Sperm structure and sperm motility of the African and Rockhopper penguins with special reference to multiple axonemes of the flagellum

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ABSTRACT

This study evaluated the semen of two penguin species from separate genera with reference to unique features in sperm structure using light microscopy and transmission electron microscopy. Ejaculates from African penguin (n = 51) and Rockhopper penguin (n = 9) contained on average more than 60% motile spermatozoa and a sperm concentration of 3274 × 10^6/ml and 1423 × 10^6/ml, respectively. The percentage progressive motility was similar for the two species as well as all the kinematics parameters. The sperm morphology of these two penguin species is almost identical and largely resembles that of non-passerine birds in terms of the filiform head, small acrosome and mid-piece containing 13 spherical mitochondria, arranged around the proximal and distal centrioles in a single helix. Apart from a shorter mid-piece, penguin sperm morphometrics were similar to other non-passerine birds. The ultrastructure of the sperm principal piece revealed the typical 9 + 2 microtubular arrangement without any outer dense fibres. An unusual feature in both African and Rockhopper penguin spermatozoa was the occurrence of multiple axonemes contained in one plasmalemma in 4% of spermatozoa. These double, triple and quadruple axonemal arrangements have not been described previously albeit multiple tails were reported in other bird species. It is unclear whether such a unique structural feature will be of any advantage for sperm motility and might rather be a result of the absence of sperm competition. Multiple axonemes found in penguin flagella could be an apomorphism that distinguish them from other bird spermatozoa.

1. Introduction

Eleven of the 18 species included in the avian order Sphenisciformes (penguins) are currently listed as threatened with extinction (vulnerable or endangered), and these 11 species are from the genera *Eudyptes*, *Megadyptes* and *Spheniscus* [1–3]. The African penguin (*Spheniscus demersus*) and northern Rockhopper penguin (*Eudyptes chrysocome moseleyi*) belong to the family Spheniscidae, and are classified as non-passerine bird species. The African penguin is endemic to southern Africa and was uplisted from vulnerable to endangered on the IUCN Red List of Threatened Species in 2010 [4]. This bird species has undergone several rapid population declines in their natural habitat, with the largest probably due to commercial fishing activities causing shifts in their prey populations [4]. A similar decline was observed in endangered Rockhopper penguins, with a 90% decrease in their numbers due to human encroachment, pollution, overfishing of prey species, and hydrocarbon exploitation [5].

The African Penguin Biodiversity Management Plan [6], recommended the development of conservation strategies for this penguin species both in the wild as well as in captive populations. One such strategy would be to understand the reproductive biology of *S. demersus*, since there is currently only limited information available for this species. At the age of four years, Sphenisciformes are sexually mature and will breed for the first time in its natural habitat. However, in captivity these birds will select a partner and start breeding much earlier, usually at two years of age [7].

An aspect of reproductive biology that could enhance conservation breeding success of both wild and captive penguin

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populations is a full understanding of the normal semen parameters and the subsequent assessment of semen quality. In order to achieve fertilization and sustain early embryonic development, it has been difficult to identify a single test capable of accurately predicting the fertility potential of an individual ejaculate [8]. Instead, the evaluation of sperm motility, concentration, vitality and morphology are considered the four most important parameters in any semen analysis [9–11]. These parameters are assumed to be under intense selection because of their close correlation with fertilization success [8]. Sperm morphology is thought to be involved in post-copulatory selection during sperm competition and fertilization [8].

Numerous studies have found a close relationship between sperm morphology and fertility, with positive correlations reported between percentage normal sperm morphology or sperm size and fertilization success [8,11,12]. Spermatozoa with structural defects (i.e. head or tail) either do not reach the oocyte or, if they do, cannot penetrate the oocyte to complete fertilization. In support, Nothling and Irons [13] reported that spermatozoa with nuclear craters and otherwise normal heads reach the oocyte and bind to the zona pellucida as well as normal spermatozoa do, but result in lower embryo quality and fertility than morphologically normal spermatozoa. Abnormal sperm morphology can also serve as an indicator of some disorders in spermatogenesis [11]. Such structural abnormalities may only be present during the early reproductive season in some birds. For instance, abnormal and immature sperm, including spermatogonia and spermatids, are more commonly observed in peregrine falcon ejaculates during the early stages of the breeding season [14].

Many techniques have been employed to determine the percentage normal and abnormal sperm morphology during semen analysis, including both light and electron microscopy [8,11,15]. Successful assessment of sperm morphology depends on a clear understanding of the methods used and different techniques involved in preparation of spermatozoa for such evaluations. For instance, a staining technique must be selected for its ability to stain spermatozoa differentially, clearly indicating the boundaries of the head, acrosome, mid-piece and tail, in order to evaluate the normality of each part [16].

Among avian species reports on sperm morphology are limited and the only bird species whose semen have been extensively studied are domestic species such as the common fowl (*Gallus domesticus*), duck (*Anas platyrhynchos*), turkey (*Meleagris gallopavo*), goose (*Anser anser*) and, more recently, Japanese quail (*Coturnix japonica*) [17–23]. While the sperm ultrastructure of more than 50 passerine birds has been investigated [42], only few reports on the detailed description of different sperm components of non-passerine species are available [25,26]. In general, sperm size and relative ratios of individual bird sperm components vary greatly and are species specific. Avian sperm morphology varies from the simple sauropsid form to a complex helical type with an exterior ribbon-like membrane and a long flagellum. Examination of sperm morphology of poultry semen by Maree [16], indicated that the mid-piece is considerably longer than that of other bird species, approximately one quarter of the head length. In non-passerine birds the mid-piece is short, but longer than in the dove and pigeon. The flagellum is long, although much shorter than in passerine birds [27].

Due to a general lack of information on Sphenisciformes sperm morphology and the importance of this parameter for determination of male fertility, we undertook a detailed investigation thereof. Our aim was to evaluate the sperm structure of African and Rockhopper penguins with special reference to sperm ultrastructural features using light microscopy and transmission electron microscopy (TEM). We considered it important to also include baseline information on semen and sperm characteristics such as sperm concentration and quantitative sperm motility. The results of this study will contribute reference values for future studies on the breeding soundness and conservation of these species.

2. Materials and methods

2.1. Study site and ethical clearance

The study was conducted at the Two Oceans Aquarium in Cape Town, South Africa, using penguins housed in a captive colony with breeding success. Ethical clearance was obtained for the collection of semen from the conservation agency, CapeNature (RES201/41), and all procedures were in accordance with ethical guidelines of the University of the Western Cape (ScPGC2013/06/10) and the National Zoological Gardens of South Africa (NZG/P13/07).

2.2. Animals

Captive-born male penguins used during this study had reached the adult moulting stage and were suspected to be fertile (aged four at start of study). Two African penguin (*Spheniscus dermersus*) and two northern Rockhopper penguins *(Eudyptes chrysocome moseleyi)* were habituated for the semen collection procedure from an early age. These males were never pair-bonded prior to the study and the only available breeding-age birds to collect semen samples from as part of an ongoing study on penguin reproductive biology. *S. dermersus* and *E. chrysocome moseleyi* belong to the same avian order Sphenisciformes and appear to be phenotypically similar. Males were housed indoors under artificial lighting at a constant temperature range (23–30 °C). The light regime was manipulated throughout the year to mimic natural seasonal changes. Penguins were given a standardized optimal fish diet that was supplemented with vitamins and minerals.

2.3. Semen collection

Semen was collected during the 2014–2016 breeding seasons using an unrestrained, cooperative method as described for the Magellanic penguin [28]. At the beginning of the breeding season, males displayed breeding behaviour including vocalizations, flipper spreading, head shaking, and body uplifting. The birds voluntarily followed their keepers to a quiet area of the indoor enclosure where the males willingly mounted the keepers' legs whilst continuing to exhibit breeding behaviour. Ejaculates were collected on a petri dish held in position close to the averted cloaca. After ejaculation, semen was deposited into a collection vial and kept at 35 °C for transport purposes until semen analysis commenced in less than 30 min after collection. A total number of 60 ejaculates (51 African penguin and 9 Rockhopper penguin ejaculates) were collected and evaluated. Each ejaculates of each species was treated, analysed and evaluated separately for the different parameters.

2.4. Evaluation of semen

2.4.1. Standard semen parameters

Standard semen parameters were analysed to assess the quantity and quality of ejaculates. Macroscopic analysis included assessment of semen colour and volume, while microscopic analysis involved measurement of sperm concentration, total motility, progressive motility and number of spermatozoa in the ejaculate. The volume was measured by aliquoting small quantities of semen into an Eppendorf tube using a micro pipette with an accuracy of 1 μl. Ham's F10 was used to dilute semen for sperm motility and concentration measurements, as it has been used extensively to